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Expression of Th1/Th2 cytokines in human blood after *in vitro* treatment with chlorpyrifos, and its metabolites, in combination with endotoxin LPS and allergen *Der p1*

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ABSTRACT: Exposure to organophosphate (OP) pesticides has been associated with respiratory symptoms and may be related to asthma; however, few studies have examined the molecular basis for these associations. Asthma and allergic disorders are characterized by elevated Th2 cytokines (IL-4, IL-5, IL-13), whereas the chronic inflammatory response in asthmatic airways is maintained by Th1 cytokine IFN-γ. The goal of this *in vitro* study was to examine the effects of OP chlorpyrifos (CPF), and its metabolites chlorpyrifos-oxon (CPO) and 3,5,6-trichloro-2-pyridinol (TCP), singly, and in combination with endotoxin lipopolysaccharide (LPS) or house dust mite *Dermatophagoides pteronyssinus* (*Der p1*) allergen, on expression of IFN-γ and IL-4, Th1 and Th2 signature cytokines, respectively. Cytokine expression was measured by ELISA and flow cytometry. Human blood cultures were treated with CPF/CPO/TCP (1–1000 μg ml^{-1}) and LPS (1.5–2.5 μg ml^{-1}) or *Der p1* (200 AU ml^{-1}) and supernatants were collected at 48 h. Pesticides CPF, CPO and TCP did not induce cytokine expression *in vitro*, while LPS and *Der p1* induced IFN-γ and IL-4 expression, respectively. Whole blood cultures treated with low doses of CPO (1 and 10 μg ml^{-1}), in combination with LPS, expressed higher levels of IFN-γ than LPS alone ($P < 0.05$). While CPO increased LPS-dependent induction of IFN-γ, CPO treatment did not alter *Der p1* induction of IL-4. The interaction between CPO and LPS, which results in an increased type 1 immune response, should be investigated further, particularly since the combination of OP pesticides and endotoxin is common in rural, agricultural communities. Copyright © 2006 John Wiley & Sons, Ltd.

KEY WORDS: cytokines; chlorpyrifos; organophosphate pesticides; house mite allergen; endotoxin; whole blood culture; immunotoxicity mixtures; humans

Introduction

Pesticides and their metabolites are ubiquitous in the environment as a result of widespread agricultural and domestic use (Ware, 2004). Organophosphate (OP) pesticides constitute 70% of all insecticide products used in the United States and chlorpyrifos (CPF) was the most widely used OP prior to 2001 when EPA restricted its residential use (EPA, 2001). However, agricultural applications of CPF are still permitted and it is applied in large quantities throughout the world (Lemus and Abdelghani, 2000).

Studies of OP pesticide-induced toxicity have focused on carcinogenicity, genotoxicity and neurotoxicity (Banerjee, 1999), whereas a systematic approach to the evaluation of pesticide immunotoxicity still is being developed and validated (Colosio *et al*., 1999; Germolec, 2004). Immunotoxic effects of OP exposure that have been reported in both humans and animals include symptoms of hypersensitivity and immunosuppression (Voccia *et al*., 1999; Galloway and Handy, 2003). For example, applicators of CPF had impaired lymphocyte mitogenesis, increased levels of auto-antibodies, increased percentages of CD26-positive, but decreased CD5-positive thymocytes (Thrasher *et al*., 2002). Rats exposed to CPF had elevated percentages of CD5 and CD8 cells (Blakley *et al*., 1999), impaired T-cell, but not B-cell, blastogenesis (Navarro *et al*., 2001). Together, these findings suggest that CPF exposure is associated with alterations in immune cell profiles that could be markers for immunotoxicity.

Recently, exposure to CPF has been linked to the onset of respiratory, asthma-like symptoms in villagers in rural China ($n = 22\,528$) exposed to OPs in occupational and environmental settings (Zhang *et al*., 2002), and to the onset of wheeze in a cohort of pesticide applicators ($n = 10\,246$) in Iowa and North Carolina, USA (Hoppin *et al*., 2002). Induction of asthma and allergic

disorders is related to T-helper-2 (Th2) cytokines (IL-4, IL-5, IL-13) (Larche *et al*., 2003; Romagnani, 1994). In addition, the Th1 cytokine IFN-γ has been associated with the chronic inflammatory response in airways of people with severe asthma (Ngoc *et al*., 2005). This information can aid in an experimental study of the relation between CPF exposure and immunologic profiles that are associated with a predisposition to asthma.

To the best of our knowledge, no data on cytokine changes associated with CPF or its metabolites have been reported for human cell culture systems. In humans, CPF undergoes oxidative desulfuration by enzymes in the liver to form the toxic, intermediate product chlorpyrifos-oxon (CPO), which is hydrolysed mainly to 3,5,6-trichloro-2-pyridinol (TCP) (Nolan *et al*., 1984; Morgan *et al*., 2004). The only reported observation related to OPs effect on cytokine production *in vitro* is with respect to fenitrothion, and the results indicate an inhibition of IFN-γ at high doses (Nakashima *et al*., 2002).

Our study examined the effects of low doses of CPF, CPO and TCP, that is representative of human exposure (Whyatt *et al*., 2003) and of potential concern for agricultural communities where OP exposure is common. Furthermore, the effects of treatment with CPF or its metabolites on cytokine production potentially can be altered in the presence of other immunogens. For example, endotoxin lipopolysaccharide (LPS) induces Type 1 cytokine expression, of which IFN-γ is the signature cytokine (Mattern *et al*., 1994), whereas house dust mite allergen *Dermatophagoides pteronyssinus* (*Der p1*) is associated with Type 2 cytokine expression such as IL-4 (Comoy *et al*., 1998; Hammad *et al*., 2001; Charbonnier *et al*., 2003). Since both LPS and *Der p1* are likely to be high in an agricultural setting (Braun-Fahrlander *et al*., 2002), the purpose of this study was to determine, in an *in vitro* system, whether exposure of human blood cultures to CPF, or its metabolites CPO and TCP, or to LPS endotoxin or *Der p1* allergen alone and in combination was associated with changes in expression of Type 1 or Type 2 cytokine production.

Materials and Methods

Table 1 summarizes 19 independent experiments that were conducted from September 2003 to October 2004 on blood samples obtained from 14 human volunteers. Experiments 1–6 were performed to optimize the protocol to detect cytokine expression by ELISA in supernatants of whole blood cultures after treatment with pesticide CPF or metabolites (CPO, TCP), endotoxin and *Der p1* allergen. Based on the results of experiments 1–6, experiments 7–14 were carried out with the same protocol to evaluate the intra- and inter-individual variability in cytokine responses to LPS and pesticides. Experiments 15–19 used the same treatment protocol to assess the

effects of house dust mite allergen *Der p1* treatment (singly and in combination with pesticides) on cytokine expression by ELISA; the results were confirmed by flow cytometric detection of intracellular Th1 (IFN-γ) and Th2 (IL-4) cytokine production.

Whole blood samples were obtained from healthy laboratory volunteers ($n = 6$; age 19–25; 3 male, 3 female) who were sampled repeatedly, for experiments 1–13, or were purchased ($n = 8$) from AllCells (Berkeley, CA) for experiments 14–19. Specimens were collected directly into vacutainers with heparin and processed within 2 h of collection.

Whole Blood and Isolated Lymphocyte Cultures

Whole blood was diluted 1:2 with 37 °C RPMI (CellGro) supplemented with L-glutamine 100 U ml^{-1}), penicillin (100 U ml^{-1}) and streptomycin (100 U ml^{-1}) and then aliquotted into 12 × 75 mm tubes (Becton Dickinson) in a 1 ml volume per tube. The tubes were placed in a culture rack, slanted at 45° to provide maximum surface area, and incubated at 37 °C, in 5% CO$_2$. At 48 h, the supernatants were collected and stored at −20 °C until further analysis. For isolated lymphocyte cultures, peripheral blood mononucleated cells (PBMCs) were isolated from fresh, heparinized venous blood by centrifugation over a Ficoll gradient, washed in 1X PBS and resuspended at 2 × 10^6 cells ml^{-1} in RPMI 1640 medium (Gibco) supplemented with 10% subject plasma, 1% penicillin and 1% streptomycin.

Treatment Protocols

Two hours after initiation of culture, cells were treated with vehicle controls (DMSO, Sigma, CO, USA), pesticide or pesticide metabolites (CPF/CPO/TCP, ChemService), lipopolysaccharide (LPS, Sigma), house dust mite antigen *Dermatophagoides pteronyssinus* (*Der p1*, Hollister-Stein, Washington, USA) or their combinations. Working concentrations of CPF/CPO/TCP (0.1–1 mg ml^{-1}) were dissolved in DMSO immediately before treatment. The lowest dose (1 µg ml^{-1}) selected for our *in vitro* experiments represented a realistic human exposure, as previously reported in human biomonitoring studies (Nolan *et al*., 1984; Whyatt *et al*., 2003) and thus, is of potential concern to agricultural communities where both OP and LPS exposures are common. The final concentration of DMSO in culture was set at 1% volume/volume since high concentrations of DMSO can inhibit cytokine production. A range of LPS (0.25–25 µg ml^{-1}) and *Der p1* (0.2–200 AU ml^{-1}) were tested; 2.5 µl and 200 AU ml^{-1} were chosen for LPS and *Der p1*, respectively, based on the observed induction of cytokines and minimal cytotoxicity.

Table 1. Summary of experiments conducted to evaluate effects of chlorpyrifos (CPF), chlorpyrifos-oxon (CPO), and (TCP) with and without endotoxin lipopolysaccharide (LPS) and house dust mite allergen *Dermatophagoides pteronyssinus* (*Der p1*) on Th1/Th2 cytokine expression

Exp	Date	Subjects	Culture		Assay		Treatments: single					Treatments: combined					
												CPF		CPO		TCP	
			WB	IL	ELI	ICS	E	A	CPF	CPO	TCP	+E	+A	+E	+A	+E	+A
1	09.02.03	✓	✓		✓		✓				✓						
2	09.11.03		✓		✓		✓			✓	✓			✓		✓	
3	09.16.03		✓		✓		✓							✓		✓	
4	10.09.03		✓		✓		✓							✓		✓	
5	10.22.03	✓	✓		✓		✓							✓	✓	✓	✓
6	12.02.03	✓	✓		✓		✓	✓								✓	✓
7	12.12.03		✓		✓		✓	✓						✓		✓	
8	03.03.04		✓		✓		✓			✓				✓		✓	
9	03.12.04	✓	✓		✓		✓		✓	✓	✓					✓	
10	05.24.04	✓	✓	✓	✓	✓	✓	✓			✓		✓				
11	05.26.04	✓	✓	✓	✓	✓	✓	✓			✓		✓				
12	06.01.04		✓	✓	✓	✓	✓	✓	✓	✓	✓						
13	06.02.04		✓	✓	✓	✓	✓	✓	✓	✓	✓		✓				
14	08.24.04	✓	✓	✓	✓	✓	✓										
15	09.08.04		✓	✓	✓	✓		✓				✓					
16	09.14.04	✓	✓		✓		✓	✓		✓		✓		✓	✓	✓	✓
17	09.22.04	✓	✓		✓		✓	✓		✓		✓		✓	✓	✓	
18	10.05.04	✓				✓	✓	✓		✓		✓		✓	✓		✓
19	10.19.04	✓						✓							✓		

EXP, experiment number; WB, whole blood; IL, isolated lymphocytes; ELI, ELISA; ICS, intracellular cytokine staining; E, endotoxin; A, allergen; CPF, chlorpyrifos; CPO, chlorpyrifos-oxon; TCP, 3,5,6-trichloro-2-pyridinol.

Viability

To determine cell viability, 20 µl of culture suspension was added to 180 µl of 0.4% trypan blue. The percent viability was determined by division of the number of live cells by the total number of cells. The positive control phorbol-myristate-acetate (PMA)/ionomycin) and LPS treatments had viabilities of 90% and 96%, respectively. Viability for CPO treatments of 1 and 10 µg ml^{-1} were 98% and 93%, respectively, and declined to 88% with a dose of 100 µg ml^{-1}. Doses of 500 and 1000 µg ml^{-1} were cytotoxic.

Cytokine ELISA

Cytokine-specific ELISA was performed with Biosource cytoset reagents in accordance with the manufacturer's instructions (Biosource, CA). Briefly, plates were coated with cytokine-specific capture antibody (5 µg ml^{-1}) and incubated overnight at 4 °C for 24 h. Standards (IL-4: 10–2000 pg ml^{-1}; IFN-γ: 10–10 000 pg ml^{-1}) and samples were added, followed by 100 µl of biotinylated anti-cytokine-detection antibody (11 µg ml^{-1}). After washes, 100 µl tetramethylbenzidine (TMB) substrate was added for 20 min. The color reaction was stopped by addition of 50 µl of 0.2 M H$_2$SO$_4$ and optical densities were read for each well with a microplate reader set to 405 nm.

Intracellular Detection of Cytokines by Flow Cytometry

Interferon-γ (IFN-γ) and interleukin-4 (IL-4) cytokines were detected in T-helper and total T-cells with fluorochrome-conjugated, monoclonal antibodies specific for T-helper cells (CD4/PerCP) or pan-T-cell (CD3/PerCP), interferon-γ (IFN-γ/FITC) and interleukin-4 (IL-4/PE), as previously described (Duramad *et al*., 2004). The percent Th1 was determined as the number of IFN-γ positive cells divided by the total number of T-helper cells, and the percent Th2 was determined as the number of IL-4 positive cells divided by the total T-helper cells.

Statistics

Student's *t*-test was used to evaluate the observed difference between culture type (whole blood versus isolated lymphocytes) and to compare the observed difference between treatment types (LPS alone versus LPS and CPO combined treatment) (Stata, Version 6.0). For the subset of five subjects with repeated observations, a mixed linear model analysis (SAS Institute, Version 9.0) that accounted for repeated experiments with the same subjects was used to evaluate differences between the treatment conditions.

Results

Induction of IFN-γ by LPS and CPO in Human Blood Cultures

Treatment with LPS alone resulted in IFN-γ levels of 3110 and 3485 pg ml^{-1} in whole blood (WB) and isolated lymphocyte (IL) cultures, respectively, from the same donor (Fig. 1). Treatment with the combination of LPS and CPO (≤100 µg ml^{-1}) resulted in IFN-γ levels of 6312 and 3540 pg ml^{-1} in WB and IL cultures, respectively. IFN-γ levels were significantly higher (78%) in WB cultures treated with the combination of LPS and CPO, compared with LPS alone ($P = 0.02$) and furthermore, WB levels were more than twice that detected in IL cultures ($P = 0.001$). This initial finding indicated that CPO, at levels that are representative of environmental exposures, could lead to an increase in LPS-dependent induction of IFN-γ and that this effect is more pronounced in WB than IL cultures. Thus, for subsequent repeat experiments with multiple donors, the optimized WB culture protocol was used. Also, the study specifically examined the effect of low doses (1 µg ml^{-1}) of CPF, CPO, and TCP which are particularly relevant to realistic exposures observed in human populations (Whyatt *et al*., 2003).
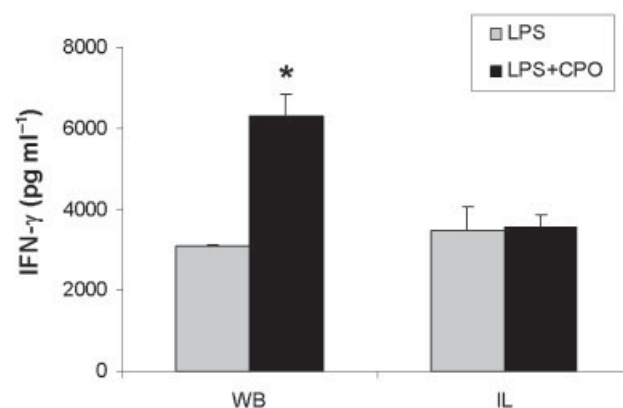

Figure 1. Supernatant levels of IFN-γ in human whole blood (WB) versus isolated lymphocyte (IL) cultures from the same donor after treatment with LPS alone or in combination with chlorpyrifos-oxon (CPO) after 48 h. Single asterisk (*) indicates significant difference between LPS and LPS + CPO treatment groups for whole blood cultures ($P = 0.02$) and significant difference between whole blood and isolated lymphocytes when treated with LPS + CPO ($P = 0.001$)
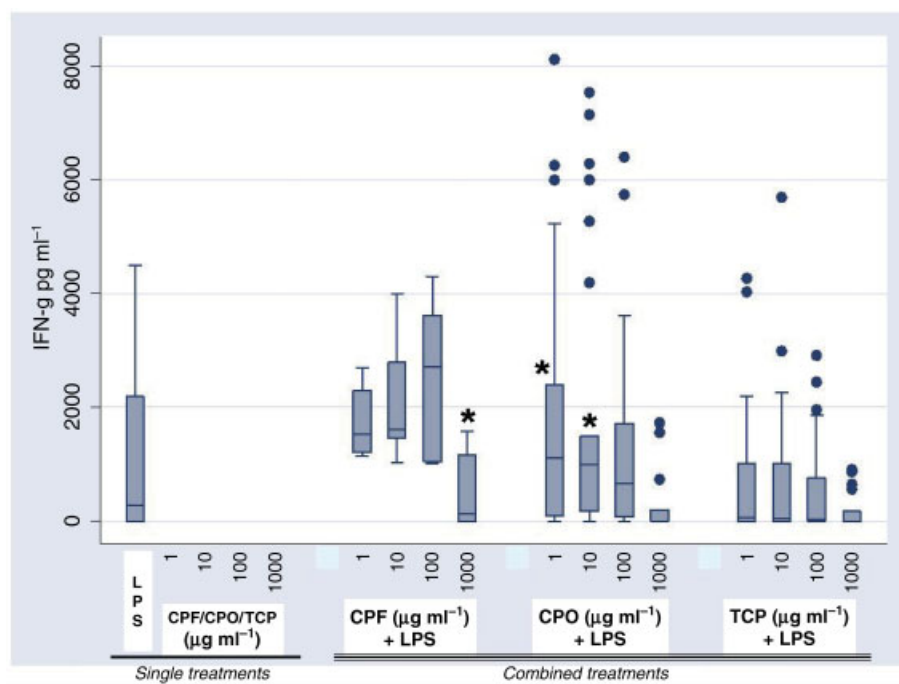
Figure 2. Supernatant levels of IFN-γ in human whole blood (WB) cultures after treatment with by CPO, TCP or CPF, singly and in combination with LPS, for five subjects sampled repeatedly over 1 year for a total of 16 experiments. A linear mixed-model statistical model was used to account for the repeated sampling of the same subject over time. Asterisks (*) indicate that the mean is significantly different from the mean of LPS treatment alone ($P < 0.05$). This figure is available in colour online at www.interscience.wiley.com/journal/jat

Combined Treatment with OPs and LPS

To examine whether the observed potentiation of LPS induction of IFN-γ was specific to CPO or can be also observed with CPF and TCP, multiple experiments were carried out with repeated blood samples from five different subjects. For LPS treatment alone the average IFN-γ level was detected by ELISA at 1363 pg ml^{-1} (Fig. 2). These experiments demonstrated a wide range of inter- and intra-individual variability in IFN-γ in response to LPS treatment over time (range 137–2507 pg ml^{-1}). Thus, a linear mixed model that accounted for both sources of variability was used to analyse the data from 16 experiments. Combined treatment with LPS with CPF at concentrations of 1, 10 and 100 µg ml^{-1} resulted in increased levels of IFN-γ: 1678, 1930 and 2179 pg ml^{-1}, respectively. The same experiment with CPO also increased IFN-γ expression to 2179, 2061 and 1648 pg ml^{-1}, respectively. Statistical analysis confirmed that two low doses of CPO (1 and 10 µg ml^{-1}), in combination with LPS, significantly increased IFN-γ expression (60% and 51%, respectively) compared with LPS alone ($P < 0.05$). Combined treatment with LPS and TCP, however, did not result in a potentiation of the cytokine induction.

At the high dose of CPF (1000 µg ml^{-1}), in combination with LPS, a significant inhibition of IFN-γ expression was observed ($P < 0.05$). This inhibition was also observed for the same dose of CPO + LPS and TCP + LPS, however, these changes were not statistically significant.

IL-4 Induction by Der p1 and Pesticides

IL-4 cytokine production was detectable in the supernatants of *Der p1*-treated cultures (200 pg ml^{-1}; 7 experiments, data not shown). However, in contrast to potentiation of IFN-γ production by CPO and LPS, the pesticide treatment in combination with *Der p1* did not affect induction of IL-4 or with two other pesticides (data not shown).

Intracellular Cytokine Production

Flow cytometric detection of intracellular cytokine expression was employed to determine whether T-helper cells were the main source of IFN-γ found in cultures from one donor after treatment with CPO and LPS. A total of 16157 lymphocytes were selected by a circular gate (Fig. 3A) for analysis of intracellular cytokine production. Of this population of lymphocytes, 5100 CD4-positive T-helper cells (31.6%) were detected by fluorescent antibody (Fig. 3B). Of these T-helper cells, less than 1% (5/5100) expressed intracellular IFN-γ in response to LPS + CPO induction (Fig. 3C). This is
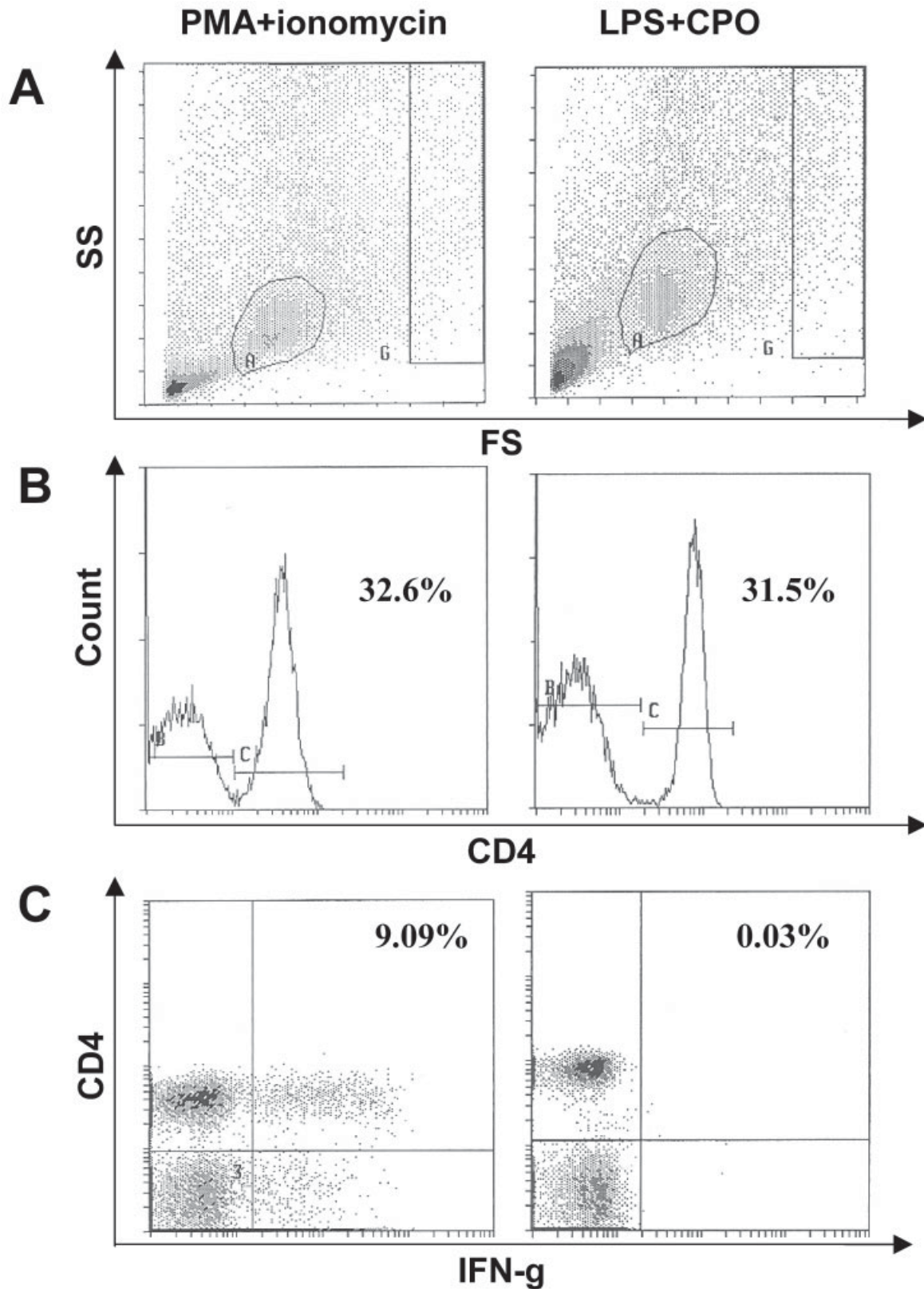
Figure 3. Flow cytometric detection of intracellular IFN-γ production by T-helper cells after treatment with CPO and LPS. In the scatter plot of all cells (A) a circular gate was placed around the live lymphocyte population, based on size (FS, forward scatter) and granularity (SS, side scatter). This lymphocyte population is acquired to (B). The percentages for CD4 antigen (of the total lymphocytes) are above each linear gate. In (C) each CD4-positive sub-group gated is examined for expression of IFN-γ. The percentages of Th1 cells are of the total CD4+ population

significantly less than the number of cells recorded after treatment with the positive control PMA/ionomycin, for which 9% (1375/5194) of T-helper cells expressed IFN-γ. Thus, T-helper cells may not be the source of IFN-γ and other cell types (e.g. CD8+ cytotoxic T-cells, macrophages, natural killer cells etc.) should be considered as potential sources of IFN-γ.

Discussion

In order to model the exposure patterns for agricultural workers and their families, blood was obtained from human volunteers and the samples exposed to the organophosphate pesticide chlorpyrifos (CPF) or its metabolites CPO and TCP, in the presence or absence of LPS or *Der p1*. Our results showed that while OP pesticides did not induce IFN-γ cytokine production in cell cultures, combined treatment with low doses of CPO (1 μg ml^{-1}) and LPS increased IFN-γ production significantly in comparison with LPS alone (Figs 2 and 3). These results suggest that the OP metabolite, at low, environmentally relevant doses, can potentiate expression of IFN-γ, the signature Th1 cytokine, *in vitro*.

In the only previous study of OPs and cytokines, Nakashima *et al.* (2002) demonstrated that the OP fenitrothion inhibited production of IFN-γ and IL-2 in a dose-dependent manner (1–500 μM). Hooghe *et al.* (2000) reported inhibition of cytokines IFN-γ with atrazine, but not mecoprop, simizine and diuron. In a separate study, pyrethroids (e.g. natural pyrethrum and synergist piperonyl-butoxide) inhibited IFN-γ and IL-4 expression in human samples obtained from atopic and non-atopic donors (Diel *et al.*, 2003). We are not aware of any other published studies that have examined the effects of combined CPO and LPS treatment on human immune function using whole blood cultures. In rats, Singh and Jiang (2003) showed that chronic exposure to low levels of the organothiophophate insecticide, acephate, enhanced responses to LPS induction of pro-inflammatory cytokines IL-1β, TNF-α, and IFN-γ. Gordon *et al.* (1997) and Gordon and Rowsey (1999) have shown in rats that CPF induction of cytokines is similar to LPS induction of inflammatory cytokines and that TNF-α, but not IL-6, is the important cytokine mediator of these inflammatory fever-like symptoms.

Although our *in vitro* findings indicate that combined LPS and CPO treatment enhanced induction of cytokines over LPS alone, they failed to show a similar relationship of *Der p1* and CPO. Dong *et al.* (1998) demonstrated that rats treated with the carbamate insecticide carbaryl, had enhanced allergic responses to *Der p1* (Dong *et al.*, 1998). Sato *et al.* (1998) also showed that the phenthoate, chlornitrofen and paraquat (but not 12 other pesticides tested) increased histamine release in mice pre-sensitized with LPS. Collectively, these finding suggest that some pesticides can augment immune responses to known immunogens LPS and *Der p1*.

The use of the whole blood culture method to test for immunomodulatory agents is becoming more common (Hermann *et al.*, 2003). This method has been used to evaluate cytokine responses to pyrogens (Hartung *et al.*, 1996) and has been proposed to screen for immunotoxic compounds (Langezaal *et al.*, 2001; Hermann *et al.*, 2003). We used both whole blood and isolated lymphocyte cultures to examine the effects of exposures to the different mixtures of cells present in each culture. The observed responses were greater using whole blood cultures. There are several advantages to using the whole blood system. First, compared with isolated lymphocytes, whole blood cultures contain a more complete mixture of the immune cells involved in generation of the immune response to an antigen and for an *in vitro* system, are more representative of an *in vivo* system. Our finding that T-helper cells are not the likely source of IFN-γ further supports this premise, since monocytes and macrophages, also producers of IFN-γ, usually are not found in isolated lymphocyte cultures. Additional advantages of whole blood cultures include the ease of culture and the low volume of blood required to initiate these cultures, a clear advantage for pediatric studies that have limited access to blood samples (Duramad *et al.*, 2004).

Our preliminary finding that CPO enhances LPS induction of IFN-γ suggests that up-regulation may occur along the LPS-Toll-like-receptor (TLR)-4 signaling pathway. CPF has been shown to up-regulate the protein kinase-C signaling (PKC) pathway (Bagchi *et al.*, 1997) and more recently, CPO has been shown to potentiate diacylglycerol-induced extracellular signal-regulated kinase (ERK 44/42) along this pathway (Bomser and Casida, 2000; Bomser *et al.*, 2002). Since PKC is a critical signaling event in oxidative stress and pro-inflammatory cytokine synthesis, increased PKC activity is a possible explanation for the increased IFN-γ observed in our experimental results. The interaction observed between CPO and LPS, which results in an increased Type 1 immune response, should be further investigated, particularly since the combination of OP pesticides and endotoxin is common in rural, agricultural communities.

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